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## IMMUNE RESPONSE OF HUMANS TO THE CIRCUMSPOROZOITE PROTEIN OF *PLASMODIUM FALCIPARUM*: LIMITED T CELL RESPONSE TO THE IMMUNODOMINANT CENTRAL REPEAT REGION

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**Abstract.** Most adults in highly malarious areas have antibodies to the repeat region of the circumsporozoite protein of *Plasmodium falciparum*. To determine if a T cell epitope on the repeat region stimulated T cell help for this antibody, we used R32tet<sub>32</sub>, a recombinant construct derived from the repeat region of the circumsporozoite protein of *P. falciparum*, to stimulate in vitro mononuclear cells from residents of an area hyperendemic for malaria. Three groups differing in the length of time they had resided in a malarious area were studied. The percentage of individuals in each group who had positive antibody responses to R32tet<sub>32</sub> increased with increased exposure to malaria. However, antibody positivity was not correlated with in vitro lymphocyte proliferation responses to the antigen. Lymphocytes from 79% of the individuals showing serum antibodies to R32tet<sub>32</sub> failed to respond in a lymphocyte transformation assay, suggesting that T cell helper activity in these individuals was based upon the recognition of a T cell epitope not located within this peptide.

Humans naturally exposed to malaria make antibodies to the repeat region of the circumsporozoite (CS) protein.<sup>1-3</sup> A recombinant DNA and a synthetic peptide subunit vaccine derived from the CS protein of *Plasmodium falciparum* have shown promise in the protection against experimental, sporozoite induced malaria in humans.<sup>4-6</sup> They both include Asn-Ala-Asn-Pro (NANP), the predominant tetrapeptide repeat on the CS protein. However, human antibody response to immunization with these vaccines has been inconsistent.<sup>4-6</sup> It is known that the murine T helper cell response to (NANP)<sub>n</sub> is severely restricted genetically,<sup>7,8</sup> and that at least one additional epitope on the CS protein carboxyterminal to the repeat region can stimulate T cells to provide help for the production of the antibody to (NANP)<sub>n</sub>.<sup>9</sup> A possible explanation for the inconsistent response of humans immunized

with these vaccines is that there is some genetic restriction of the human T cell response to (NANP)<sub>n</sub>. To investigate this possibility, we studied adults living in malarious areas of Indonesia. Although we found a high prevalence of serum antibodies to (NANP)<sub>n</sub>, our results suggest that T lymphocyte response to this antigen is restricted, and that other epitopes on the CS protein or on sporozoites are important to the induction of T cell help for the production of antibodies to (NANP)<sub>n</sub>.

### MATERIALS AND METHODS

#### Study populations

Volunteers in the study were adult residents of three villages on the Indonesian island of Irian Jaya. The villages of Mapurujaya and Kwamki Lama are located in highly malarious areas of the coastal lowlands. The village of Tembaga-pura is located in a mountainous area where ma-

laria transmission has never been demonstrated; however, the residents do travel occasionally to the malarious lowlands. Giemsa stained thick blood smears from all volunteers were screened for malaria infections. Controls included 1 Indonesian and 7 American volunteers who had no history of malaria.

#### *Blood sampling and processing*

For each volunteer, a sample of blood was obtained for serum. Sera were separated by centrifugation and immediately frozen in liquid nitrogen. Another sample of blood was drawn into heparin, diluted into an equal volume of RPMI 1640 with  $\text{NaHCO}_3$  and carried 12–14 hr at ambient temperature to a central laboratory for immediate processing. Peripheral blood mononuclear cells (PBMC) were purified on Ficoll Hypaque. Trypan blue staining, as well as concanavalin A (Con A) and pokeweed mitogen (PWM) stimulation were employed for all samples to confirm cell viability and function.

#### *Cellular proliferation studies*

The recombinant DNA construct, R32tet<sub>32</sub>,<sup>8</sup> was purified by ammonium sulfate precipitation, diafiltration, ion exchange chromatography, and reversed phase chromatography. Cellular responses to this antigen were studied by lymphocyte transformation assay (LTA). Purified PBMC were suspended in RPMI 1640 at a concentration of  $2.5 \times 10^6/\text{ml}$  and triplicate 100  $\mu\text{l}$  volumes were placed in flat-bottomed, 96-well tissue culture plates (Linbro Chemical Co., Hamden, CT). Cells were stimulated with Con A (Sigma, St. Louis, MO) at 5  $\mu\text{g}/\text{ml}$ , PWM (Gibco, Grand Island, NY) at a dilution of 1:100 and R32tet<sub>32</sub> at final concentrations from 5 ng/ml to 5  $\mu\text{g}/\text{ml}$ , added in 100  $\mu\text{l}$  volumes of RPMI. Incubation was carried out in a humidified 37°C chamber with 5%  $\text{CO}_2$ . One  $\mu\text{Ci}$  of  $^3\text{H}$ -thymidine (New England Nuclear, Boston, MA) was added after 72 hr of culture for Con A and 120 hr for PWM and R32tet<sub>32</sub>. The cells were harvested using a multiple automated sample harvester 18 hr after adding the thymidine, and counted in a Packard TriCarb scintillation counter. For each volunteer, stimulation indices were calculated by dividing the counts per min (cpm) of stimulated cells by the cpm of the same individual's cells cultured without antigen. A stimulation index

$\geq 2$  SD above the mean control value was considered positive.

To test for possible direct cytotoxic effects of R32tet<sub>32</sub> on lymphocytes in vitro, an aliquot of cells from each volunteer from Mapurujaya was cultured as above without R32tet<sub>32</sub>, and a second aliquot was cultured in the presence of 5  $\mu\text{g}/\text{ml}$  of the antigen. No isotope was added. On day 7, cultures were checked for viability by Trypan blue dye exclusion. Paired cell cultures were then compared for the percentage of viable cells remaining.

#### *Detection of antibodies*

For each volunteer whose cells were tested by LTA, a paired serum was screened by ELISA for the presence of IgG class antibody against R32tet<sub>32</sub>. Control sera were from the same controls as in the LTA procedure. The ELISA was done using 96-well round-bottomed plates coated with R32tet<sub>32</sub> (2.0  $\mu\text{g}/\text{ml}$ ), provided by R. Wirtz, Walter Reed Army Institute for Research. Coated plates were blocked with 0.5% BSA, and incubated with 100  $\mu\text{l}$  of sera (1:100 in PBS/Tween) overnight at 4°C. Plates were washed and treated with 100  $\mu\text{l}$  of goat antisera to human IgG (1:1,000) (Tago, Burlingame, CA) for 2 hr at 37°C. The plates were then washed and 100  $\mu\text{l}$  of peroxidase conjugated rabbit anti-goat IgG (1:1,000) (KPL, Gaithersburg, MD) was added for 2 hr at 37°C. Color was developed with o-phenylenediamine. The color reaction was stopped at 30 min, and an  $\text{OD}_{492} \geq 2$  SD above the mean value in American and Indonesian controls was considered positive. All ELISA values represent the mean of duplicate runs of triplicate well measurements.

## RESULTS

Antibody to the R32tet<sub>32</sub> antigen was found in the sera of 27/39 (69%) of the volunteers from Mapurujaya and 23/40 (58%) of those from Kwamki Lama. Volunteers from Tembagapura, whose exposure to malaria was much less, showed an antibody positivity rate of 9/38 (24%). The comparison of ELISA results with LTA results revealed no correlation between serum antibody positivity and cell mediated responses to the R32tet<sub>32</sub> antigen. Fifty-seven of the antibody-positive individuals were studied by LTA. Lymphocytes from 12 of these individuals (21%) were

TABLE I  
Volunteers showing both positive cellular and humoral responses to R32tet<sub>32</sub> in vitro

Location	Volunteer	cpm*			SI	OD <sub>492</sub> †
		Cont.	R32	Δ		
Mapurujaya	1103	402	890	488	2.21	0.194
	1118	1,308	3,209	1,901	2.45	0.054
	1157	4,208	10,939	6,731	2.60	0.051
	3032	199	1,555	1,356	7.80	0.181
	3052	105	6,431	6,326	61.25	0.060
	3109	134	559	425	4.17	0.207
	3222	307	1,185	878	3.86	0.059
	3327	586	1,865	1,279	3.18	0.069
	3382	762	2,039	1,277	2.68	0.073
	$\bar{x}$				10.02	0.105
Kwamki Lama	5155	336	2,470	2,134	7.35	0.101
Tembagapura	D.M.	211	692	481	3.28	0.097
	J.M.	188	682	494	3.63	0.052
	$\bar{x}$				3.46	0.075

\* Cont. = incorporation of <sup>3</sup>H-thymidine, cells cultured without antigen; R32 = highest cpm, cells cultured with 5 ng-5 μg/ml R32tet<sub>32</sub>; Δ = R32 - cont.; SI = R32/cont.

† ELISA OD<sub>492</sub> reading for IgG antibodies that recognize R32tet<sub>32</sub>, 1:100 serum dilution. Positive serum = OD<sub>492</sub> ≥ 0.042.

stimulated to proliferate in vitro by 5 days of culture with the antigen (Table I). The 45 non-responders showed a stimulation index (SI) of  $1.38 \pm 0.36$  (mean  $\pm$  SD). Ten of these 57 individuals were found to be positive for *P. falciparum* infections at the time of sampling, and in no case did cells from blood smear-positive individuals proliferate in response to R32tet<sub>32</sub>. Lymphocytes from nonimmune Indonesian and American controls failed to respond to in vitro stimulation with R32tet<sub>32</sub> at any of the concentrations tested ( $SI = 1.23 \pm 0.41$ ). Cells from all 57 of these individuals as well as from all controls showed strong in vitro mitogenic responses, and the 7-day cytotoxicity test using R32tet<sub>32</sub> antigen revealed no significant difference in the percentage of viable cells remaining.

#### DISCUSSION

Evidence presented in this paper clearly indicates that although residents of an area endemic for *P. falciparum* produced IgG class antibodies to the central repeat region of the *P. falciparum* CS protein, in vitro cellular proliferation responses to this antigen were not correlated with antibody positivity. Lymphocyte proliferation to R32tet<sub>32</sub> was observed in only 12 of 57 individuals who had antibodies to R32tet<sub>32</sub>. There are several possible explanations for this disparity. The immunosuppression known to be associated with *P. falciparum* infections<sup>9, 10</sup> may have lim-

ited the response. None of the 10 individuals with documented falciparum parasitemia had a positive blastogenic response to R32tet<sub>32</sub>, but these observations may have been due to chance. It is possible that others had undetected low grade parasitemia which led to specific malaria immunosuppression. However, all 10 of these individuals showed strong responses to Con A and PWM in vitro, responses that were not different from those shown by volunteers from the same group who were negative for *P. falciparum*. An alternate explanation for these observations is that a positive lymphocyte proliferative response to R32tet<sub>32</sub> represents an in vitro correlate of an in vivo cellular immune response that lowers the incidence of malaria.

It is also possible that the antigen concentrations may have been above or below the optimum range for this assay. Although in vitro proliferation responses were noted with lymphocytes from different individuals at all antigen concentrations from 5 ng-5 μg, other nonresponders might have responded to concentrations outside of this range.

However, the most likely explanation is that T cell epitopes outside the repeat domain provide T cell help for production of CS protein antibody to the repeat region, as in mice.<sup>7</sup> If this is the case in humans, *P. falciparum* sporozoite vaccines that incorporate such epitopes may prove to be more immunogenic than the first generation repeat region vaccines.

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